In Vitro Recombinant Receptor/Reporter Gene Assays for Assessing the Estrogenic and Dioxin-Like Activity of Compounds and Complex Mixtures

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Abstract

In vitro recombinant receptor/reporter gene assays have been developed to assess and rank the potency of substances and complex mixtures suspected of possessing estrogenic and/or dioxin-like activity. The assays take advantage of the receptor-mediated mechanism of action of estrogenic and dioxin-like compounds. The Environmental Estrogen Bioassay (E2 Bioassay) uses a Gal4-human estrogen receptor chimeric construct (Gal4-HEGO) and a Gal4-regulated luciferase reporter gene (17m5-G-Luc) which can be transeintly transfected into any established cell line and has been stably transfected into HeLa cells. The E2 Bioassay exhibits 40- to 50-fold induction following treatment with 1 nM 17β-estradiol (E2) and has a detection limit of 5 pg of E2/ml. The Ah receptor (AhR) Bioassay uses Hepa 1c1c7 wild type cells stably or transiently transfected with a DRE-regulated luciferase reporter gene and the endogenously expressed AhR and Ah receptor nuclear translocator (ARNT). This bioassay has a detection limit of 16 pg of TCDD/ml and exhibits approximately 10-fold induction in luciferase activity following treatment with 1 nM TCDD. The utility of the assays are demonstrated by examining the activity of pulp and paper mill black liquor, chloro-S-triazine-derived compounds and naringenin, a plant bioflavonoid.

Introduction

Several *in vivo* and *in vitro* assays have been developed to assess the estrogenic or dioxin-like activity of individual compounds or complex mixtures. These assays use a variety of endpoints including increased in target organ weight[1)], enzyme and recombinant reporter gene induction[2,3,4,5,6,7)], ligand binding [8,9,10)], increased protein expression[4,11)] and cell proliferation or differentiation[12,13)]. Advantages of *in vitro* bioassays include cost effectiveness, accurate prediction of potential toxic impact, the capability of handling large sample numbers and they also provide an indication of the possible mechanism of action. Moreover, they account for synergistic, antagonistic and additive interactions which may occur between compounds within complex mixtures that are not considered by using

conventional chemical residue analysis. In this study, we demonstrate the utility of recombinant receptor/reporter gene bioassays by detecting the presence of estrogenic and dioxin-like activity in pulp and paper mill black liquor, a recovered by-product in the pulping industry and assessing the estrogenic potency of naringenin, a plant bioflavonoid and two commonly used triazine herbicides, atrazine and simazine.

Materials and Methods

Materials. Black liquor, a recovered by-product in the pulping industry, was obtained from a modernized bleached kraft mill located in southern Ontario (supplied by Dr. K. Burnison, National Waters Research Institute, Burlington, Ontario). Approximately 3% of black liquor is lost to the sewer system which carries the waste to the primary and secondary treatment facilities of the mill before the final effluent is discharged to the receiving waters. All other chemicals were of the highest quality available from commercial sources.
Reporter Gene Assays. The MCF-7 human breast cancer cells, Hepa 1c1c7 wild type mouse hepatoma cells and stably transfected HeLa cells were maintained as previously described[14,15]. Cells were transiently transfected using the calcium phosphate co-precipitation technique[16] and reporter gene assays were performed as described in Brasier *et al.*[17] or using the Luciferase Assay System (Promega). The stable transfectants were prepared as previously described[2,18].

Results

TCDD treatment of Hepa 1c1c7 wild type cells transiently transfected with a DRE-regulated reporter gene (pGudLuc1.1) exhibited a dose dependent increase in luciferase activity (Figure 1). Based on these results, the AhR bioassay has a detection limit of 16 pg of TCDD/ml and a 10-fold maximum induction. Cells treated with serially diluted black liquor also exhibited a dose dependent induction in reporter gene activity.

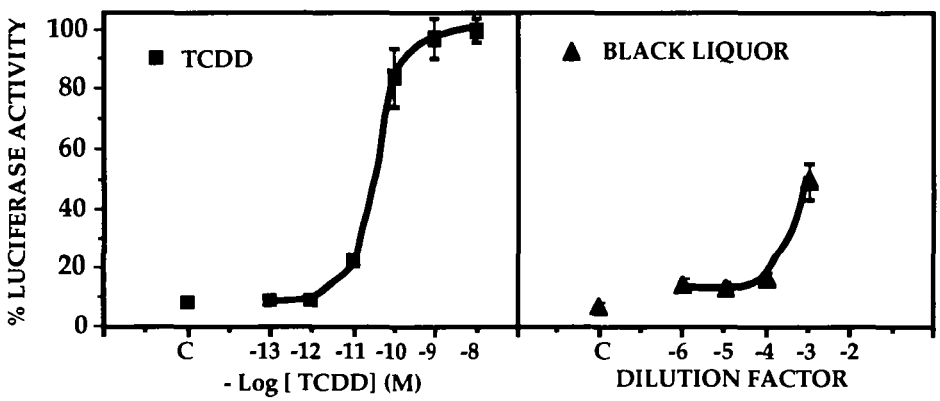

Figure 1. Effect of TCDD and pulp and paper mill black liquor on the induction of a DRE-regulated luciferase reporter gene transiently transfected into Hepa 1c1c7 wild type cells.

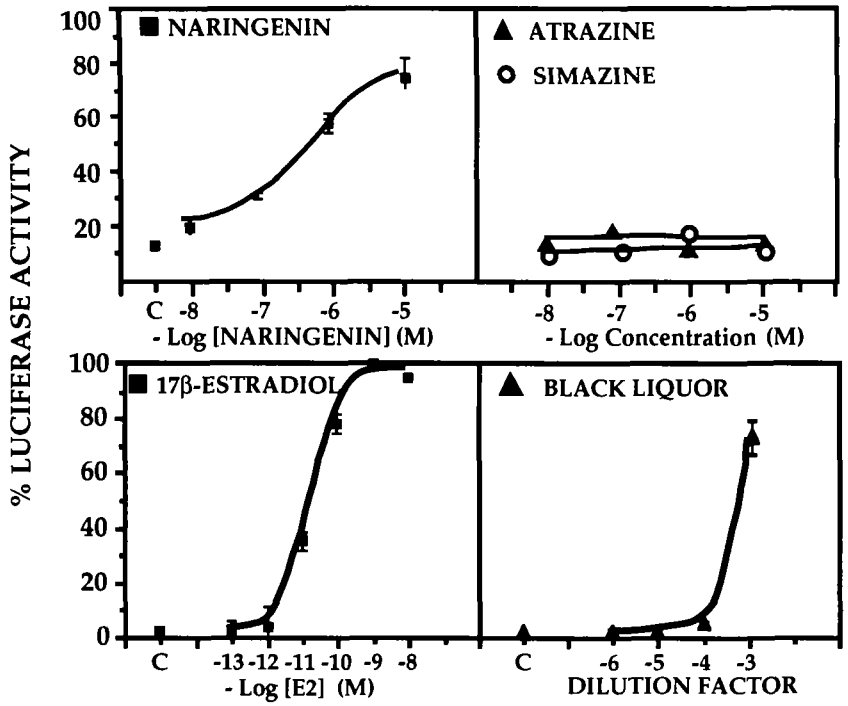

Figure 2. Effect of 17β-estradiol (E2), pulp and paper mill black liquor, atrazine, simazine and naringenin on the induction of a Gal4-regulated luciferase reporter gene, 17m5-G-Luc.

Figure 2 summarizes the the ability of E2, black liquor, atrazine, simazine and naringenin to induce Gal4-estrogen receptor chimera-mediated induction of the Gal4-regulated reporter gene, 17m5-G-Luc. E2 (1 nM) maximally induced luciferase activity 40- to 50-fold, exhibiting an EC_{50} of 20 pM. Black liquor and naringenin also exhibited significant estrogenic activity. In contrast, atrazine and simazine did not induce luciferase activity, at concentrations as high as 10 μM.

Discussion

This report demonstrates the utility of *in vitro* recombinant receptor/reporter gene bioassays to detect and assess the estrogenic and dioxin-like activity of compounds and complex mixtures. Several *in vivo* and *in vitro* bioassays have been previously used however, most exhibit modest responsiveness and can be induced by a number of divergent mechanism which may lead to false-positive results. Moreover, many of the endpoints that are monitored are laborious and time consuming which limits the number of samples that can be examined in a timely manner. The E2 Bioassay and AhR bioassays address these limitations and provides a sensitive method which is amenable to automation.

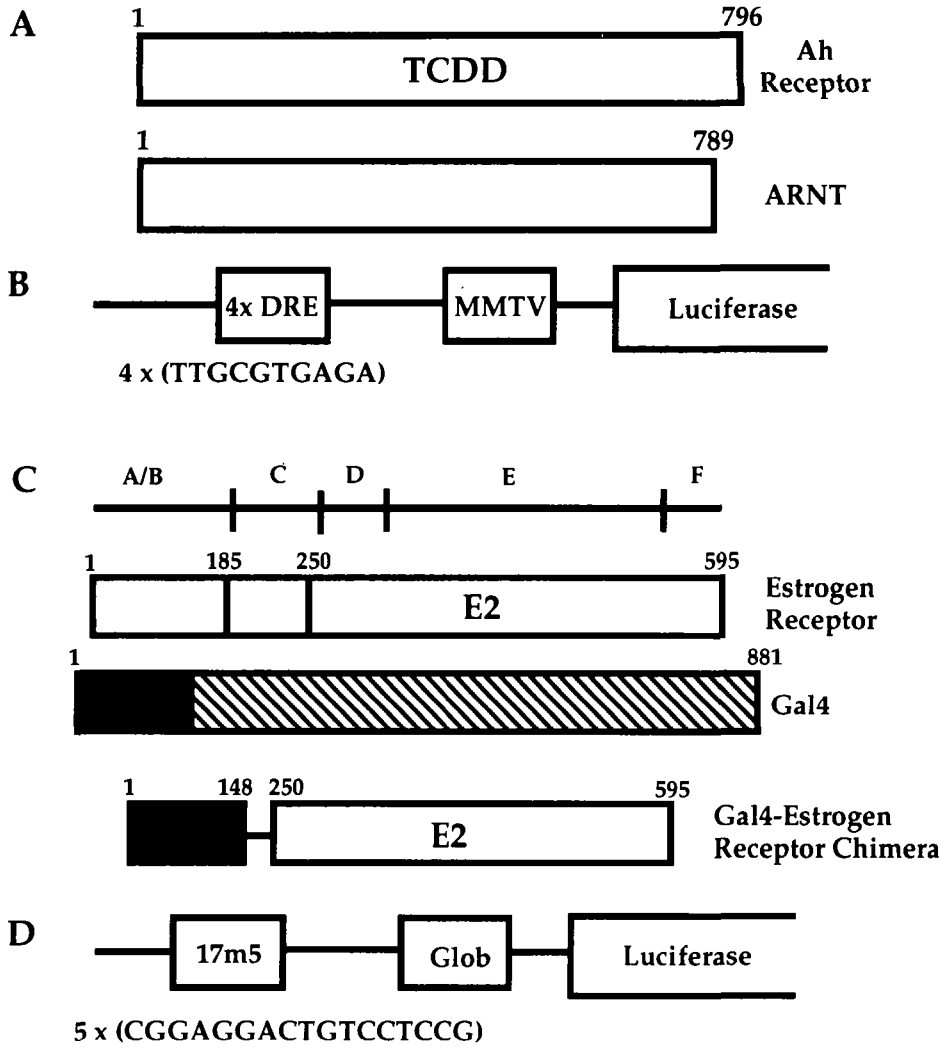

Figure 3. Components of the AhR and E2 Bioassays. A - Endogenously expressed AhR and ARNT, B - the DRE-regulated reporter gene, pGudluc1.1, C - The Gal4 - human estrogen receptor chimera, D - the Gal4-regulated reporter gene (17m5-G-Luc).

The AhR Bioassay uses Hepa 1c1c7 wild type cells which endogenously express AhR and ARNT and a transiently transfected DRE-regulated reporter gene (pGudluc1.1) to assess the dioxin-like activity of a compound or complex mixture. In addition, a stably transfected cell line has also be prepared. The advantage of this bioassay is that induction of luciferase activity can only occur through the AhR; therefore, other mechanisms such as mRNA stability or oxidative stress, which have been reported to influence AHH activity are

excluded. Consequently, the specificity and diagnostic nature of this bioassay is improved which is an important consideration when examining complex environmental and industrial samples. Induction of pGudluc1.1 activity following treatment of transiently transfected Hepa 1c1c7 wild type cells demonstrates that black liquor possess ligands with dioxin-like activity. However, previous studies have shown that this induction is not due to polychlorinated dibenzo-*p*-dioxins or dibenzofurans and therefore, further research is necessary to identify the inducing agent [15].

The central component of the E2 Bioassay is the Gal4-human estrogen receptor chimera (Gal4-HEGO) which is composed of the Gal4 DNA binding domain linked to the ligand binding domain of the ER (i.e. domains D, E and F, see Fig. 3c). Gal4 is a DNA binding yeast transcription factor involved in the regulation of galactose utilization and has no known mammalian homologue. It recognizes and binds to a specific response element referred to as a 17mer (Fig. 3d) which, in isolation, is sufficient to confer Gal4-dependent induction to a recombinant reporter gene. The Gal4-regulated reporter (17m5-G-Luc) consists of five tandem 17mer response elements upstream of the rabbit β-globin promoter linked to the firefly luciferase cDNA reporter gene. Luciferase activity is easily measured and provides a rapid, sensitive, non-radioactive and non-toxic assay to monitor the induction potency of compounds or complex mixtures [17]. Since there is no known mammalian equivalent to Gal4 and reporter gene induction is strictly regulated by the 17mer response elements, any increases in 17m5-G-Luc activity can only occur via Gal4-HEGO, thus demonstrating that the sample possesses estrogenic activity which is mediated by the estrogen receptor.

Recent studies have suggested that black liquor, atrazine, simazine and naringenin possess estrogenic activity [19, 20, 21]. The results presented in Figure 2 clearly demonstrate that black liquor and naringenin possess estrogenic activity that is mediated by the estrogen receptor. In contrast, no induction in luciferase activity was observed in the E2 Bioassay following treatment of the cells with atrazine or simazine, thus the estrogenic effects elicited by these compounds are not ER-mediated. However, these results do not exclude that a possible interaction could occur between chloro-S-triazines and ER-mediated responses.

This study demonstrates the utility of recombinant receptor/reporter gene bioassays in the assessment of compounds and complex mixtures which are suspected of possessing estrogenic and dioxin-like activity. The assays can be used: (i) to complement conventional chemical analysis by indicating the presence of compounds that may not be detected by GC-MS analysis due to the lack of appropriate standards, (ii) in the assessment of the potential toxic potency of the sample, (iii) to indicate possible mechanisms of action, (iv) as economical monitoring systems to assess remediation efforts when detailed chemical residue analysis is not required, (v) as a screen for identify complex mixture fractions that possess activity during the isolation and identification of the active agents and (vi) in determining toxic equivalency factors for environmental estrogens and dioxin-like compounds. Moreover, the availability of stable transfectant cell lines should facilitate the incorporation of these assays into most laboratories.

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